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Human Coronavirus NL63 and 229E Seroconversion in Children▽

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In 2004, the novel respiratory human coronavirus NL63 (HCoV-NL63) was identified, and subsequent research revealed that the virus has spread worldwide. HCoV-229E is a close relative of HCoV-NL63, and infection with either virus can lead to the hospitalization of young children, immunocompromised persons, and the elderly. Children infected with HCoV-NL63 often develop croup, with obstruction of the airway. In this study we investigated at which age children are confronted for the first time with an HCoV-NL63 infection and, thus, at which age they seroconvert to HCoV-NL63 positivity. We designed a recombinant HCoV-229E and a recombinant HCoV-NL63 nucleocapsid protein enzyme-linked immunosorbent assay and performed a sero-epidemiology survey on longitudinal and cross-sectional serum samples. The longitudinal serum samples were collected from 13 newborns, and data for those newborns were available from multiple time points spanning a period of at least 18 months. For the cross-sectional survey we tested serum samples of 139 children, including newborns to children 16 years of age. In examinations of the longitudinal serum samples we observed that all of the children had maternal anti-NL63 and anti-229E antibodies at birth that disappeared within 3 months. Seven of the 13 children became HCoV-NL63 seropositive during follow-up, whereas only 2 became HCoV-229E seropositive. The serology data of the cross-sectional serum samples revealed that 75% and 65% of the children in the age group 2.5 to 3.5 years were HCoV-NL63 and HCoV-229E seropositive, respectively. We conclude that on average, HCoV-NL63 and HCoV-229E seroconversion occurs before children reach the age of 3.5 years.

Coronaviruses (CoVs) are enveloped, positive-strand RNA viruses belonging to the family *Coronaviridae* (12). The genomic RNA is 27 to 32 kb in size and is capped and polyadenylated. The virions have a unique morphology, with extended, petal-shaped spikes that give the virus a projection that resembles a crown (in Latin, *corona*) under the electron microscope (12). All CoVs possess a common genome organization in which the replicase gene encompassing the 5′ two-thirds of the genome is comprised of two overlapping open reading frames, ORF1a and ORF1b. The structural gene region, which covers the 3′ third of the genome, encodes the canonical set of structural protein genes in the order 5′-spike (S)→envelope (E)→membrane (M)→nucleocapsid (N)-3′. The S and N proteins are the proteins most abundantly expressed during virus infections, and both S and N proteins induce an immune response (12). CoVs are classified into three groups based on phylogenetic and serological relationships (12). Groups 1 and 2 consist of various mammalian CoVs, whereas avian viruses cluster in group 3.

Nowadays, there are five different human CoV (HCoV) species known to exist, all of which are associated with respiratory tract infections in humans. HCoV-229E (a group 1 CoV) and HCoV-OC43 (group 2) were discovered in 1966 and 1967, respectively (9, 14). Both HCoVs were identified as the causative agents of the common cold in humans by experimental inoculation of healthy adult volunteers (1–3, 11, 17). Almost 40 years later the severe acute respiratory syndrome outbreak emerged. The causative agent was identified as a novel member of the group 2 CoVs (5). Over 8,000 severe acute respiratory syndrome-CoV infections were reported during the peak period of the 2002 and 2003 outbreak, with a mortality rate of 10% (5). After it was determined that highly pathogenic HCoVs can evolve, efforts to identify and characterize new HCoVs increased. This resulted in the identification of two new family members in 2004 and 2005, HCoV-NL63 (group 1) and HCoV-HKU1 (group 2), respectively (21, 24). Infections with either HCoV-HKU1 or HCoV-NL63 can lead to hospitalization of young children and of elderly and immunocompromised patients (21). In addition, HCoV-NL63 is associated with croup, which is a common manifestation of lower respiratory tract infections, with a peak occurrence in the first two years of life (22). Globally, approximately 10% of all upper and lower respiratory tract infections of hospitalized children are caused by HCoV-229E, HCoV-OC43, HCoV-HKU1, and HCoV-NL63 (8, 20).

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Clinical studies have indicated that HCoV-NL63 infections frequently occur in children below the age of 3 years (22). These studies encompassed populations of children who were hospitalized due to respiratory tract infections. This seems to indicate that HCoV-NL63 infections in children are likely to lead to hospitalization; however, we hypothesize that HCoV-NL63 infections are common during childhood and that only a minor fraction of infections require hospitalization of children. Retrospective investigation of all HCoV-NL63 infections in children is possible with a serological assay such as an enzyme-linked immunosorbent assay (ELISA). The relationship between age and frequency of infection can be monitored by measurement of HCoV-NL63 antibody titer increases (seroconversion). The N protein of CoVs is conserved within species, it is immunogenic, and it induces one of the strongest immune responses of all structural proteins (25). The N protein of HCoV-NL63 shares only 42% amino acid identity with the N protein of the closest relative of HCoV-NL63, HCoV-229E (16); therefore, an N protein ELISA is likely to be species specific.

We developed an HCoV-NL63 N protein ELISA and determined that this assay is species specific. We observed no cross-reactivity between HCoV-NL63 N-directed antibodies and HCoV-229E N-directed antibodies, thus confirming the specificity of the ELISA. Subsequently, we performed seroepidemiological surveys with longitudinal and cross-sectional serum samples obtained from newborns and children up to 16 years old, respectively, to determine at which age children seroconvert to HCoV-NL63 positivity and are thus confronted for the first time with an HCoV-NL63 infection.

MATERIALS AND METHODS

Serum for longitudinal and cross-sectional surveys. Human serum specimens from newborns were collected at the Department of Medical Microbiology, Academic Medical Center, Laboratory of Experimental Virology, University of Amsterdam, Amsterdam, The Netherlands. All children were born to human immunodeficiency virus type 1 (HIV-1)-positive mothers. Serial samples were collected during a follow-up period of at least 18 months and stored at −80°C. All 13 children remained HIV-1 RNA negative and became HIV-1 seronegative during the follow-up period.

Human serum specimens from newborns and children up to 16 years old were collected at the Department of Medical Microbiology, Academical Medical Center, Laboratory of Clinical Virology, University of Amsterdam, Amsterdam, The Netherlands. Serum samples were obtained between 1999 and 2003 and were stored at −80°C. All serum samples were heat inactivated at 56°C for 30 min.

Preparation of recombinant HCoV-NL63 and HCoV-229E N protein expression constructs. HCoV-NL63 RNA was isolated and reverse transcribed as described previously (15). The cDNA was used as a template for generation of a full-length 1,134-nucleotide N gene with the primer combination 5′ NL63_Nexp (5′-CACCGCTAGTGTAAATTGGGC-3′) and 3′ NL63_Nexp (5′-TTAATGC AAAACCTCGTTGAC-3′). The pTRE-HN plasmid (a kind gift from Volker Thiel) (18) was used as the template for amplification of the full-length 1,170-nucleotide N gene of HCoV-229E with the primer combination 5′ 229E_Nexp (5′-CACCGCTACAGTCAAATGGGCT-3′) and 3′ 229E_Nexp (5′-TTAGTTT ACTTCATCAATTAT-3′). Amplification of the N genes was performed with *Pfx* polymerase (Invitrogen). Amplified N gene fragments were cloned using pET100/D-Topo vector (Invitrogen). The sequences of the generated pET100_NL63 and pET100_229N plasmids were determined and shown to be 100% identical to the virus reference sequences (in GenBank) of HCoV-NL63 (Amsterdam-01; NC_005831) and HCoV-229E (Inf-1; NC_002645), respectively.

Expression of HCoV-NL63 and 229E N proteins. Expression of recombinant N proteins of HCoV-NL63 and HCoV-229E was determined by transformation of 10 ng of plasmid in the chemically prepared competent *Escherichia coli* BL21-derived strain Rosetta 2 (Novagen). Vector coding for the recombinant LacZ protein (by use of plasmid pET100/D/LacZ) (Invitrogen) was included as a control. Overnight cultures of transformed bacteria containing either pET100_229N, pET100_NL63N, or pET100_LacZ plasmid were inoculated into Luria broth medium, supplemented with 1% glucose, carbenicillin (10 μg/ml), and chloramphenicol (17.5 μg/ml). Cultures were grown to the exponential phase prior to induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 5 h. Recombinant proteins were purified with nickel-nitrilotriacetic acid agarose (Qiagen), and protein concentrations were determined with a Micro bicinchoninic acid protein assay (Pierce).

N protein ELISA. Ninety-six-well ELISA plates (Greiner Bio-one) were coated overnight at 4°C with 3 μg/ml of expressed recombinant N protein of HCoV-NL63 or HCoV-229E or LacZ protein (negative control). The proteins were diluted in 0.1 M carbonate buffer (pH 9.6). Unspecific binding sites were blocked with phosphate-buffered saline–0.1% Tween 20 (PBST) supplemented with 5% skim milk (Fluka) for one hour at room temperature (RT). Longitudinal and cross-sectional serum samples were diluted 1:200 and 1:100, respectively, in PBST containing 1% skim milk and incubated on the plate for 2 h at RT. After a washing, alkaline phosphatase-conjugated anti-human immunoglobulin G Fcγ-tail antibody (Jackson Immunoresearch) diluted (1:1,500) in 1% skim milk–PBST was added. Following 1 h at RT, the plates were washed and signal was developed with 50 μl of Lumi-Phos Plus (Lumigen). Measurements were done with a Glomax 96 plate luminometer (Promega). All serum samples were tested in duplicate. In the study with cross-sectional serum samples, a cutoff value was used. This value was the mean from the levels for the 6- to 12-month-old children as obtained by use of either HCoV-NL63 or HCoV-229E ELISA.

N protein competition ELISA. Human serum samples were diluted (1:200) in PBST containing 1% skim milk, and twofold serial dilutions (ranging from 0 to 50 μg/ml) of expressed recombinant N protein of HCoV-NL63, N protein of HCoV-229E, or LacZ protein were added. The mixtures were briefly homogenized by vortexing prior to incubation for 1 h at RT. No centrifugation was performed. Following the preincubations, the samples were measured by HCoV-NL63 or HCoV-229E ELISA as described above.

Statistical analysis. Calculations were performed using Prism software version 5 (Graphpad). The median 50% inhibitory concentration (IC_{50}) of the soluble HCoV-NL63 N, HCoV-229E N, and LacZ protein competitor in the competition ELISA was calculated by the nonlinear regression method, with variable slope. Comparison of longitudinal results from the cumulative incidence values for HCoV-NL63 and HCoV-229E seropositivity time points was done with Kaplan-Meier survival analyses; statistical significance was tested using a log-rank (Mantel-Cox) test. Comparison of the HCoV-NL63 and HCoV-229E analyses with cross-sectional serum samples was performed with a nonparametric Mann-Whitney U test to determine whether there was a statistically significant difference between the values obtained for the frequency of seroconversion to HCoV-NL63 and HCoV-229E positivity.

RESULTS

Comparison of the N protein sequences of HCoV-NL63 (YP_003771) and those of its closest relative, HCoV-229E (NP_073556), revealed that these proteins share only 42% similarity. We hypothesized that the difference on the amino acid level is sufficiently high to ensure the usefulness of a recombinant HCoV-NL63 N protein ELISA specific for HCoV-NL63 N protein-directed antibodies. To verify the specificity, we performed a competition ELISA with coated HCoV-NL63 N protein and incubated several HCoV-NL63 antibody-positive samples with serial dilutions of LacZ, HCoV-NL63 N, or HCoV-229E N proteins. To exclude sample-to-sample variation, we performed the competition ELISAs for four different serum samples. For all four samples we observed that incubation with the homologous protein (HCoV-NL63 N protein) did inhibit the HCoV-NL63 ELISA (IC_{50} values ranging between 9 and 43 μg/ml), whereas incubation with the heterologous proteins did not (IC_{50} values of >50 μg/ml for both HCoV-229E N and LacZ protein). Thus, in the competition assay we observed that only HCoV-NL63 N protein preincubation diminished the signal in the HCoV-NL63 N ELISA. Incubation with HCoV-229E protein or LacZ in the HCoV-NL63 ELISA

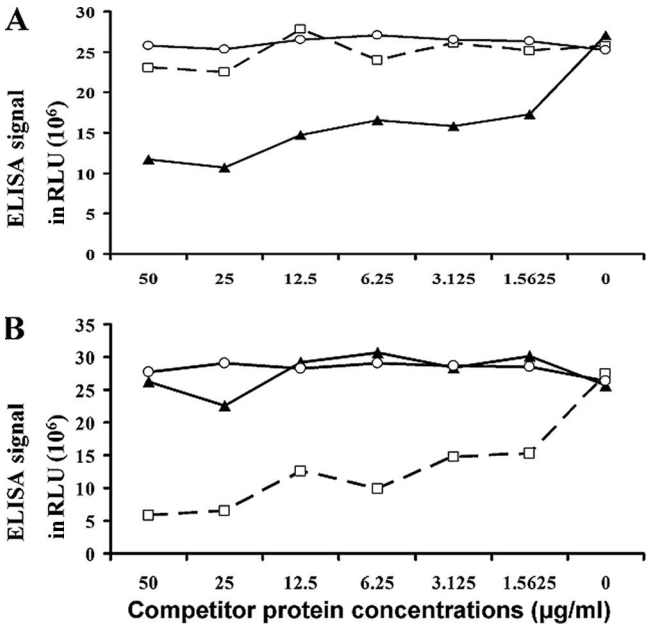

FIG. 1. Lack of cross-reactivity between antibodies directed to HCoV-NL63 N protein and those directed to HCoV-229E N protein. Serum of an adult known to be positive for HCoV-NL63 and HCoV-229E was diluted (1:200) and preincubated with serial dilutions of proteins. (A) Competition between soluble HCoV-NL63 N protein (closed triangles, continuous line), HCoV-229E N protein (open squares, dashed line), and LacZ protein (open circles, continuous line) in an HCoV-NL63 N protein ELISA. (B) Competition between soluble HCoV-NL63 N protein (closed triangles, continuous line), HCoV-229E N protein (open squares, dashed line), and LacZ protein (open circles, continuous line) in an HCoV-229E N protein ELISA. RLU, relative luminescence units.

had no effect, while HCoV-229E preincubation did inhibit an HCoV-229E N ELISA (shown for serum sample 1 in Fig. 1). Thus, no antigenic cross-reactivity between HCoV-NL63 N antibodies and HCoV-229E N antibodies was observed.

We aimed to investigate the age at which a child incurs a first HCoV-NL63 infection, and we therefore followed newborn children and determined the age at which antibody titers to HCoV-NL63 rise. We had access to sequential serum samples of newborn children that were followed until the age of approximately 1.5 years (ranging from 18 months to 29 months). Serum samples were collected at approximately months 0, 1, 3, 12, and 18. The first thing that was noted was the high level of antibodies at birth. All children carried maternal HCoV-NL63 N antibodies. The level of these antibodies decreased to very low levels within a few months. Seroconversion to HCoV-NL63 positivity during follow-up was seen for 7 of the 13 children (Fig. 2, panels C, G, H, I, J, K, and M). To determine whether seroconversion to HCoV-229E positivity occurs as frequently as seroconversion to HCoV-NL63 positivity, the same longitudinal set of samples was tested using an HCoV-229E-specific N protein ELISA. As with the HCoV-NL63 analysis, we observed that all of the children carried maternal HCoV-229E antibodies at birth and that the level of antibodies decreased within the first months of life. Only 2 of the 13 children seroconverted to HCoV-229E positivity (Fig. 2, panels G and L) during follow-up. Interestingly, one child presented a sequential increase of levels of antibodies to both HCoV-229E and HCoV-NL63 at months 16 and 27, respectively (Fig. 2, panel G). Five of the 13 children did not seroconvert to HCoV-NL63 or HCoV-229E positivity during follow-up. To investigate whether seroconversion to HCoV-NL63 occurs significantly earlier or more frequently than seroconversion to HCoV-229E, we determined the cumulative incidence (Kaplan-Meier) of seroconversion for each virus (Fig. 3). The statistical analysis revealed, however, that the difference in the frequencies of infection by each virus is not statistically significant ($P = 0.08$).

To investigate the frequency of infection of both viruses for older children, we collected a total of 139 serum samples from newborns and children up to 16 years old and performed a cross-sectional survey. We observed that the majority (64%) of infants less than 6 months of age had N protein-directed antibodies against both viruses (Fig. 4). These N protein-directed antibodies were most probably antibodies passed on from the mother. The numbers of seropositive individuals in the age category of 6 to 12 months were low for both HCoV-NL63 and HCoV-229E (33% and 22%, respectively). These percentages changed only slightly in the age group of 1 to 1.5 years: the number of individuals seropositive for HCoV-229E increased to 36%, whereas for HCoV-NL63 the number decreased to 29%. At the age of 1.5 to 3.5 years, most of the children became infected by HCoV-NL63 and HCoV-229E. The percentages of seropositive children increased almost twofold to 65% for HCoV-229E and 75% for HCoV-NL63. For children beyond the age of 3.5 years we observed that the vast majority carried antibodies directed to HCoV-NL63 and HCoV-229E (Fig. 4). As with the longitudinal survey, we did not observe a significant difference between HCoV-NL63 and HCoV-229E in frequencies of infection.

DISCUSSION

Here we show that HCoV-NL63 infections are common during childhood and that the majority of seroconversion to HCoV-NL63 positivity occurs before children reach the age of 3.5 years. This finding is comparable to what is known for other respiratory viruses, such as human metapneumovirus, respiratory syncytial virus, and human bocavirus. The approximate ages for seroconversion for these viral pathogens differ, and seroconversion seems to peak between the ages of 5 to 10 years, 2 to 5 years, and 2 to 6 years for human metapneumovirus, respiratory syncytial virus, and human bocavirus, respectively (6, 7). We observed that all of the children in our study who were older than 10 years are seropositive for HCoV-NL63, a result similar to those obtained with the previously mentioned viral pathogens (6, 7). In conclusion, HCoV-NL63 circulates among the entire population of children, and HCoV-NL63 plays an important role in acquired childhood infections.

The antibody specificity directed toward viral proteins determines which virus-specific antigen can be used in a serological assay. For HCoV-NL63, results have recently been published indicating that human serum samples contain antibodies directed against full-length recombinant expressed HCoV-NL63 N protein (23). Vlasova et al. reported that HCoV-NL63 N protein-directed antibodies displayed no antigenic cross-reactivity with the N protein of nonhuman CoVs (23). How-

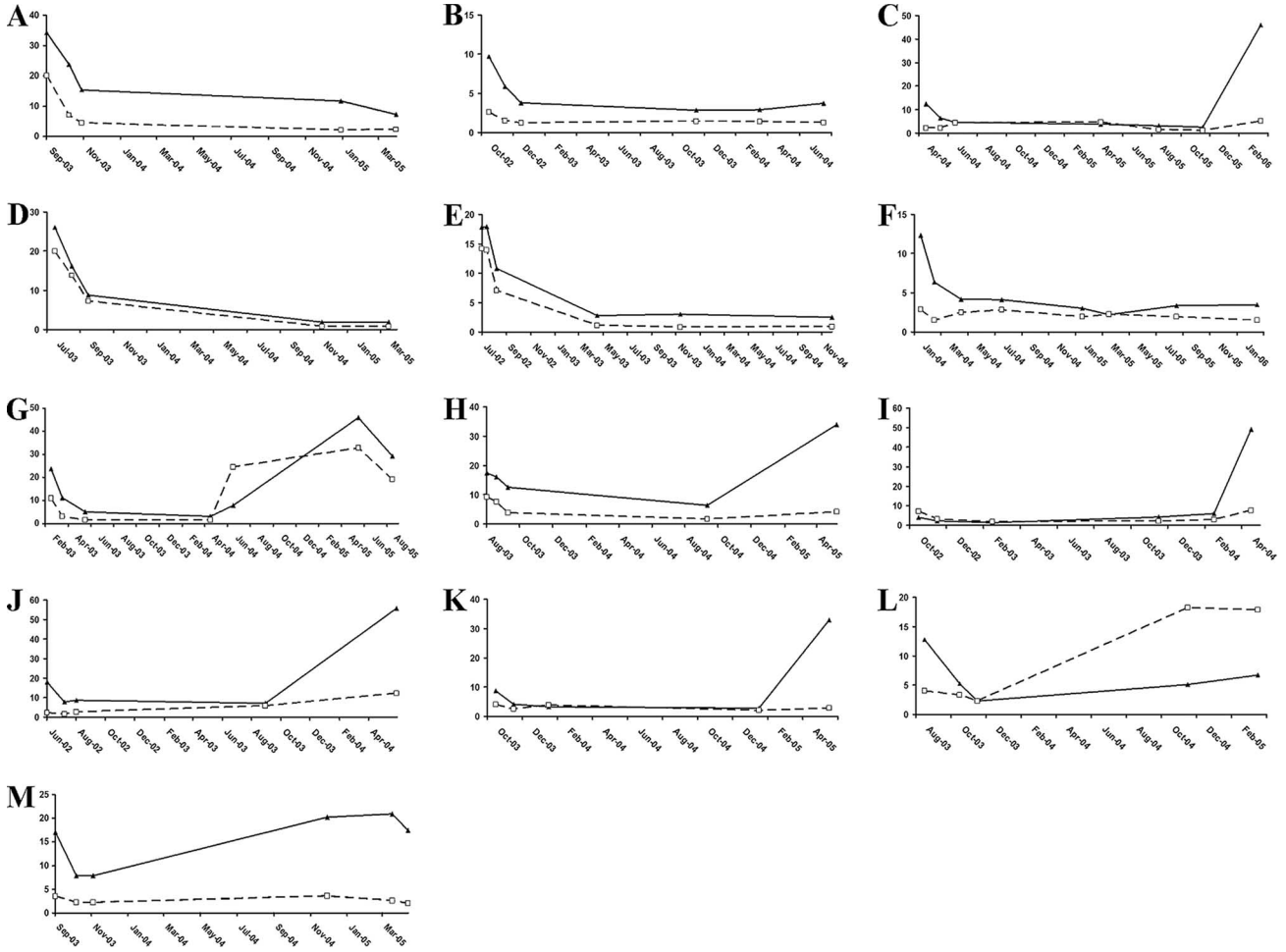

FIG. 2. HCoV-NL63 and HCoV-229E N protein-directed antibody levels in follow-up serum samples from 13 children. Each graph (A through M) represents the longitudinal profiles of levels of HCoV-NL63 N protein (closed triangles, continuous line)- and HCoV-229E N protein (open squares, dashed line)-directed antibody for a single child. The measured antibody levels are indicated as relative luminescence units (10^6) on the *y* axis. The follow-up period is plotted on the *x* axis.

ever, they did not determine whether HCoV-NL63-directed N protein antibodies could cross-react with full-length recombinant expressed HCoV-229E N protein. The CoVs' N protein-directed antibodies do not have neutralizing potential due to the location of the N proteins inside the enveloped virion (12); thus, the presence of antigenic cross-reactivity can be determined only by performing a competition ELISA and not with a neutralization assay. With the competition assay we observed no detectable antigenic cross-reactivity between HCoV-NL63

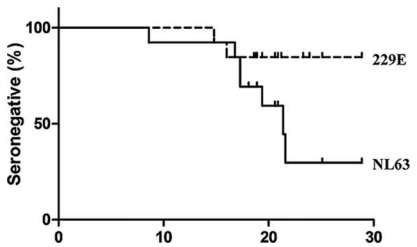

FIG. 3. Cumulative levels of incidence of HCoV-NL63 and HCoV-229E infections. The Kaplan-Meier survival analysis was performed using values representing the cumulative levels of incidence of the percentages of seronegative individuals (*y* axis) plotted against time (in months; *x* axis). Seroconversion to HCoV-NL63 positivity is presented as a continuous line; seroconversion to HCoV-229E positivity is presented as a dashed line. The time point of seroconversion was calculated by taking the midpoint between the last seronegative and the first seropositive time points.

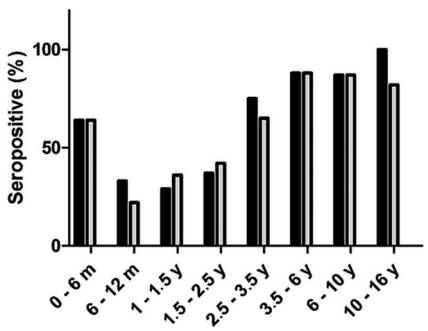

FIG. 4. Percentages of HCoV-NL63- and HCoV-229E-seropositive results in different age groups. The percentages of HCoV-NL63 (black bars)- and HCoV-229E (gray bars)-seropositive individuals were monitored using serum samples obtained from children of various ages. m, months, y, years.

N protein-directed antibodies and HCoV-229E N protein-directed antibodies. A similar finding has recently been published by another research group (13). Therefore, the utilization of HCoV N proteins in an ELISA provides a convenient tool for analyzing the seroepidemiological profiles of HCoV-NL63 and HCoV-229E.

The analysis of longitudinal serum samples from children allowed us to simultaneously measure the levels of HCoV-NL63- and HCoV-229E-directed antibodies directly after birth and with a follow-up period of at least 18 months. In all children we detected high levels of maternal HCoV-NL63 and HCoV-229E N protein-directed antibodies at birth, although the levels of maternal antibody titers differed between newborns. These antibody levels decreased to low levels within a period of 3 months and remained low until subsequent infection. Five of the 13 children remained HCoV-NL63 and HCoV-229E seronegative during follow-up. We investigated whether the levels of maternal antibodies could be used to predict a seronegative outcome during follow-up but found no correlation between the level of ELISA signal at birth and seroconversion. In addition, we tested the antibody levels in the mothers before and at birth to investigate whether low antibody titers in the mother could predict seroconversion, but we again found no correlation (data not shown).

The first seroepidemiologic study of HCoV-NL63 and HCoV-229E in children was reported by Hofmann et al. (10). They determined whether children carry neutralizing antibodies against HCoV-NL63 and HCoV-229E S glycoprotein. The neutralization assays were performed by challenging pseudoviruses carrying the HCoV-NL63 or HCoV-229E S glycoprotein with human serum samples (10). This resulted in the observation that children under the age of 1.5 years do not carry neutralizing antibodies against the S glycoproteins from either of the HCoVs. However, the number of individuals older than 1.5 years carrying HCoV-NL63-neutralizing antibodies increased, whereas for HCoV-229E this number remained low (10). We observed, however, that the number of children with HCoV-NL63-directed antibodies was equal to that of children with HCoV-229E-directed antibodies. Shao et al. also found the same incidences of HCoV-229E and HCoV-NL63 infections (19). They used a part of the C-terminal region of the N protein as an antibody capture antigen in an ELISA (19). Antibodies directed to HCoV-NL63 and HCoV-229E in children of 1 year and older were frequently detected. Thus, the findings by Shao et al. and our findings are in contradiction with the findings by Hofmann et al. The fact that Hofmann et al. measured seropositivity by using the HCoV-229E S protein of the reference strain may explain the differences in findings. For HCoV-229E, it has been determined that the S protein of the reference strain is different from those of the recent circulating strains (4); therefore, it is possible that the reference strain S protein cannot be recognized by the HCoV-229E S antibodies that are induced by current circulating strains. The HCoV-NL63 S protein used by Hofmann et al. was obtained recently (2003), which may explain why HCoV-NL63 S-directed antibodies were more frequently found than HCoV-229E S-directed antibodies. We also used the HCoV-229E and HCoV-NL63 reference strains to amplify the N gene and express the protein; however, unlike the S protein, the N protein is conserved over time (4).

In the past, most of the HCoV-229E and HCoV-OC43 serological data and details on clinical manifestations were obtained from infection trials with adult volunteers (1–3, 11, 17). The antibody levels of each volunteer were measured before entry in a trial, and all volunteers were HCoV-229E and HCoV-OC43 seropositive, although the antibody levels differed (3). The volunteers with high antibody levels developed no or fewer clinical symptoms after HCoV-229E or HCoV-OC43 inoculation, whereas those with low antibody levels did develop normal clinical symptoms after inoculation (3). Volunteers could be reinfected with either HCoV-229E or HCoV-OC43, because the antibody titers dropped within 1 year to the levels measured before participation in the volunteer trials (3). In a similar manner, HCoV-NL63 reinfections might also occur, given the seroepidemiological similarity to HCoV-229E in children. However, whether this is the case could only be determined by performing trials with volunteers, but such trials would raise ethical issues regarding the unknown pathogenicity of HCoV-NL63. Alternatively, these results could be obtained by monitoring the antibody levels in healthy adults whose longitudinally collected serum samples are available. This will allow investigation of the levels of HCoV-NL63-directed antibodies over time, which will reflect whether reinfection of HCoV-NL63 occurs.

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